

Efficient and inducible production of human interleukin 6 in Chinese hamster ovary cells using a novel expression system

Yingpei Zhang¹, Yoshinori Katakura¹, Hideya Ohashi² & Sanetaka Shirahata¹

¹ Graduate School of Genetic Resource Technology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-81, Japan; ² Pharmaceutical Laboratory, Kirin Brewery Co. Ltd., Miyahara, Takasaki, Gunma 370-12, Japan

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Abstract

High level and inducible production of human interleukin 6 (hIL-6) was achieved using a novel expression system in Chinese hamster ovary (CHO) cells. In this system, the transcription of hIL-6 gene under the control of PhCMV*-1 promoter composed of tetracycline operator sequences and a minimal promoter is activated by a chimeric transactivator (tTA) composed of tetracycline repressor and transactivating domain of VP16 protein of herpes simplex virus. The transcription of tTA gene, which is also under the control of PhCMV*-1 promoter, is activated by itself *via* a positive feedback cycle. The expression of both genes is further enhanced by potentiating the VP16 transactivating domain of tTA transactivator with pX protein of hepatitis B virus. In the presence of tetracycline, the tTA transactivators can not bind to PhCMV*-1 promoter, therefore, the expression of hIL-6 and tTA gene is suppressed, and the pX will not activate basal transcription. In the absence of tetracycline, tTA transactivators bind to PhCMV*-1 promoter and activate efficient transcription of hIL-6 and tTA gene, and the transcription is further enhanced by pX *via* VP16 transactivating domain. Using this strategy, we isolated a clone (UX1) producing hIL-6 at a rate about 1425 ng/10⁶ cells/day. Furthermore, the hIL-6 production is stringently regulated by tetracycline. This results suggested a novel strategy to establish highly efficient, inducible and cell type independent recombinant protein production system by using an artificial promoter to recruit transactivators and coactivators which can synergistically activate transcription.

Abbreviations: CHO – Chinese hamster ovary; CMV promoter – immediate early gene promoter of human cytomegalovirus; hIL-6 – human interleukin 6; pX – X protein of hepatitis B virus; VP16 – VP16 protein of herpes simplex virus

Introduction

It is a common strategy to use a strong promoter to control the transcription of target gene in mammalian recombinant protein production system (Cockett *et al.*, 1990). For example, CMV promoter derived from the regulation region of immediately early gene of human cytomegalovirus (CMV) is widely used for this purpose. This promoter contains *cis*-sequences recognized by various transcription factors including NF- κ B, CREB and AP1. Because the activity of these transcriptional factors is regulated by various cellular signals, the promoter may possess only very low tran-

scriptional activity in some cells such as lymphocytes where the activity of certain transcriptional factors is suppressed. CMV promoter is very strong in Chinese hamster ovary (CHO) cells, however, the promoter is apparently still subjected to certain negative regulation by cellular signalling network, as the expression of the gene under the control of CMV promoter can be greatly increased by enhancing the Ras mediated signal transduction (Seto *et al.*, 1996).

The transcriptional activity of a promoter is mainly determined by the interaction of the transcriptional factors recognizing the *cis*-sequences in it. Although the activity of transcriptional factors is usually regulated

by cellular signals, the regulation domain is, however, separable from the transactivating domain in most transcriptional factors.

The transactivating domain of herpes simplex virus VP16 protein is one of the strongest identified so far. When the chimeric GAL4-VP16 transactivator binds to a promoter containing *cis*-sequences recognized by GAL4 domain of the transactivator, it can efficiently stimulate transcription of the downstream gene. The DNA-binding transactivator-activated transcription can be further enhanced by a number of cellular protein factors called coactivators. The coactivator does not bind DNA themselves, but acts as the adaptor between DNA-binding transactivators and basic transcriptional machinery (Ge *et al.*, 1994; Berger *et al.*, 1992). One of the examples is PC4, which can significantly enhance the transcription activated by various transactivating domains but has little effects on the basal transcription from the minimal promoter in the absence of transactivators (Ge *et al.*, 1994).

The model of transcriptional activation mechanism suggested a strategy to develop a novel mammalian expression system. By expressing and targeting an artificial transactivator lacking the regulation domain to a minimal promoter and using a coactivator to potentiate the transactivating domain, it is possible to establish a novel system which enables to produce recombinant proteins efficiently in various types of cells.

The tetracycline inducible expression system has become a useful tool in gene regulation study (Gossen *et al.*, 1992). The system consists of two elements: one is a chimeric transactivator (tTA) composed of the tetracycline repressor derived from tetracycline resistance operon in *Escherichia coli* transposon Tn10 and the transactivating domain derived from VP16 protein. The other is a tetracycline responsive promoter (PhCMV*-1) composed of the tetracycline operator sequences and a minimal promoter. When the VP16 transactivating domain binds to PhCMV*-1 promoter through the interaction between the tetracycline repressor domain and the tetracycline operator sequences, it will efficiently stimulate the transcription from PhCMV*-1 promoter. The binding of tTA transactivator to PhCMV*-1 promoter is stringently regulated by tetracycline. In the absence of tetracycline, tTA transactivators are capable of binding to PhCMV*-1 promoter and stimulating transcription. The presence of tetracycline prevents the binding, therefore, suppresses the transcription. This distinct feature of tetracycline inducible expression prompted us to adapt it for recombinant protein production.

In the original tetracycline inducible expression system, tTA gene expression is controlled by CMV promoter. We expected that replacing the CMV promoter with PhCMV*-1 promoter might bring about several advantages. First, it will make the system cell type independent because the PhCMV*-1 promoter is not subjected cellular signal regulation as CMV promoter. Second, it will enable to efficiently activate the tTA gene expression through a positive feedback cycle (Figure 1), producing sufficient amount of tTA transactivators to efficiently activate the PhCMV*-1 promoter controlling the target gene expression. Finally, it will allow to easily isolate clones with relatively high cellular tTA protein level by first cloning in the presence of tetracycline then screening in the absence of tetracycline. Because cells with high tTA level grow slowly, using constitutive CMV promoter to direct its expression makes it difficult to isolate this population (Gossen *et al.*, 1992).

The pX protein of hepatitis B virus (HBV) has a capacity to stimulate transcription of a variety of viral enhancer-promoter units as well as cellular genes *via* a mechanism similar to cellular coactivators (Haviv *et al.*, 1995). It neither binds DNA directly (Gilman, 1993) nor activates basal promoter activity (Cross *et al.*, 1993). In transient expression experiments, pX protein was found to potentiate VP16 transactivating domain over 10 folds. It is therefore very attractive to examine the effect of pX on the recombinant protein production in the tetracycline system.

Here we reported that high level, inducible production of human interleukin 6 was achieved by using tTA transactivators to activate the transcription of both tTA and target gene and pX to potentiate the VP16-dependent transcription.

Materials and methods

Vectors and cell lines

The hIL-6 expression vector pUIL6-8 (Figure 2A) was constructed by inserting the human IL-6 cDNA fragment with EcoRI-BamHI ends (0.65 kb) into the downstream of PhCMV*-1 promoter in pUHD10-3 (a kind gift from Dr. Bujard; Gossen *et al.*, 1992). The tTA expression vector pUTA-1 (Figure 2B) was constructed by replacing the XhoI-EcoRI fragment containing the CMV promoter in pUHD15-1 (a kind gift from Dr. Bujard; Gossen *et al.*, 1992) with the XhoI-EcoRI fragment containing PhCMV*-1 promot-

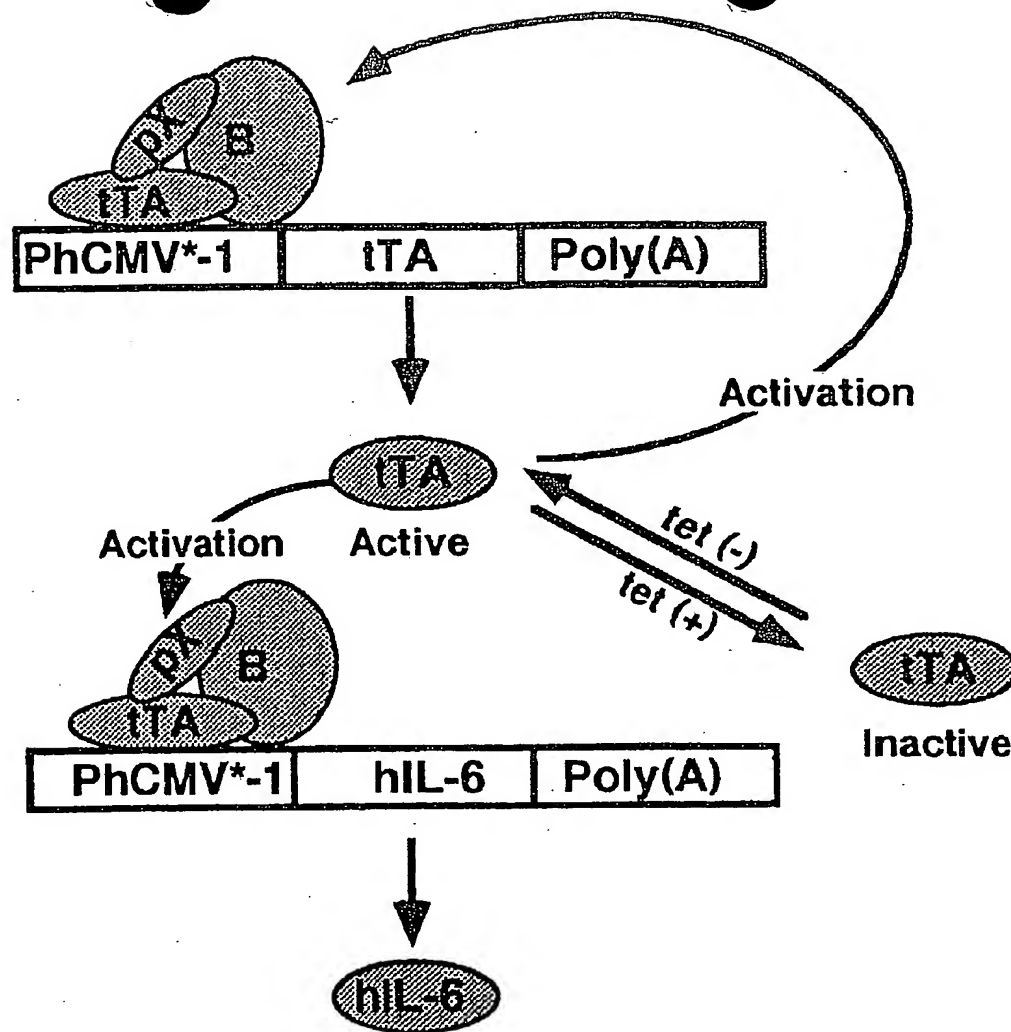


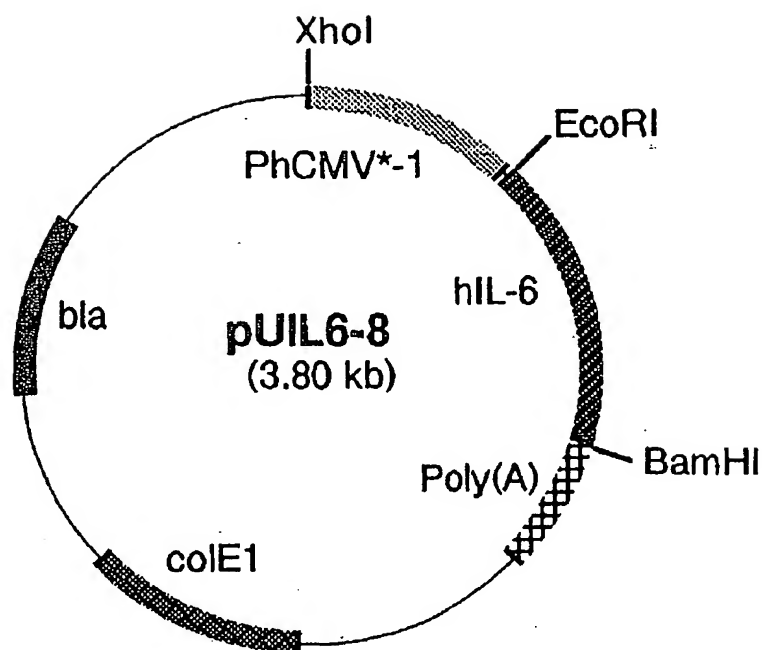
Figure 1. Principle of the expression system. In the absence of tetracycline, the minimal promoter in the PhCMV*-1 promoter can direct basal transcription of the tTA gene. The leaky expressed tTA transactivators will activate the tTA gene expression through a positive feedback cycle (upper). The high cellular tTA transactivator level ensures efficient transcription of the hIL-6 gene (lower). The tTA transactivator mediated transcription is enhanced by pX. In the presence of tetracycline, however, the expression of both genes is suppressed because tTA transactivators can not bind to the PhCMV*-1 promoter and pX can not activate transcription without VP16 transactivating domain.

er derived from pUHD10-3. The DHFR deficient CHO cell line DXB11 used as host cells were maintained in Dulbecco's Modified Eagle Medium (DME; GIBCO BRL, Gaithersburg, MD, USA) supplemented with 100 nM hypoxanthine (H) and 16 nM thymidine (T) and 10% fetal bovine serum (FBS, Biowhittaker, Walkersville, Maryland, USA). About 3×10^5 CHO cells were co-transfected with pUTA-1 (6 μ g), pUIL6-8 (6 μ g) and pSV2/bsr (2 μ g; Funakoshi, Tokyo, Japan) with calcium phosphate co-precipitation method (Chen *et al.*, 1988). The cells were first selected with blasticidin S (5 μ g ml⁻¹; Funakoshi, Tokyo, Japan) in the presence of 2 μ g ml⁻¹ tetracycline (tetracycline hydrochloride; Wako Chemicals, Osaka, Japan). The cells resistant to blasticidin S were then cloned with cloning cylinder and limiting dilution method. Screen-

ing of hIL-6 expression clones were carried out in tetracycline free medium.

The pX gene was introduced into host cells by co-transfecting with pX expression plasmid pECE-flagX (a kind gift from Dr. Y. Shaul) and pCMVD, a plasmid carrying a neo gene under the control of SV40 early promoter (unpublished) with calcium phosphate co-precipitation method. The cells were first selected with G418 (500 μ g ml⁻¹, Funakoshi, Tokyo, Japan) in tetracycline free medium, then cloned with limiting dilution method. The clones were expanded in tetracycline free medium and their hIL-6 production rates were determined.

(A)



(B)

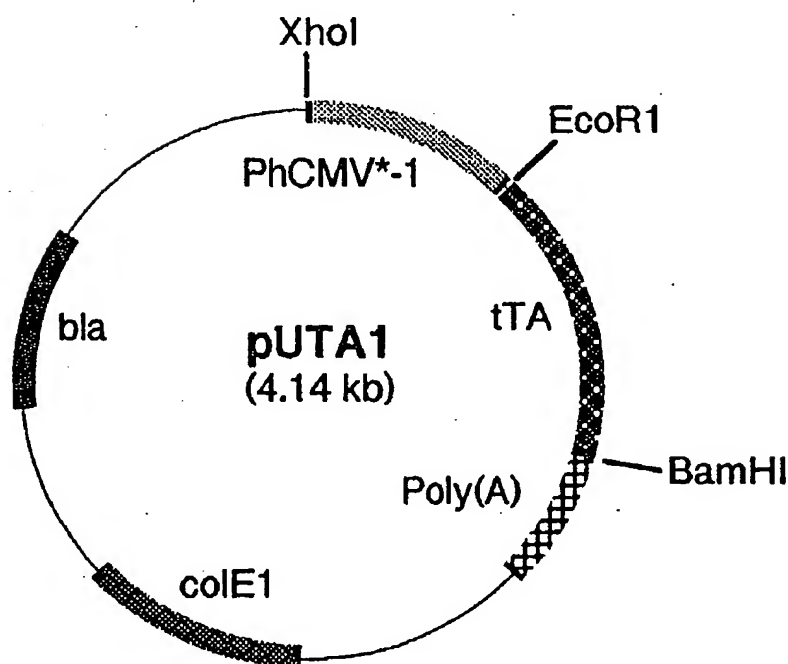


Figure 2. Vectors used in the system. (A) hIL-6 expression vector. (B) tTA expression vector.

IL-6 assay

The production of hIL-6 was determined with enzyme-linked immuno-adsorbent assay (ELISA). A microtitration plate was first coated with rabbit anti-goat IgG (H&L) antibody (Zymed, San Francisco, CA, USA),

then with anti-hIL-6 polyclonal antibody (goat-IgG, R&D Systems, Minneapolis, MN, USA). After incubated with sample solution, the plate was then incubated with a biotinylated mouse monoclonal anti-hIL-6 antibody (Genzyme, Cambridge, MA, USA) followed by streptavidin horseradish peroxidase conjugate.

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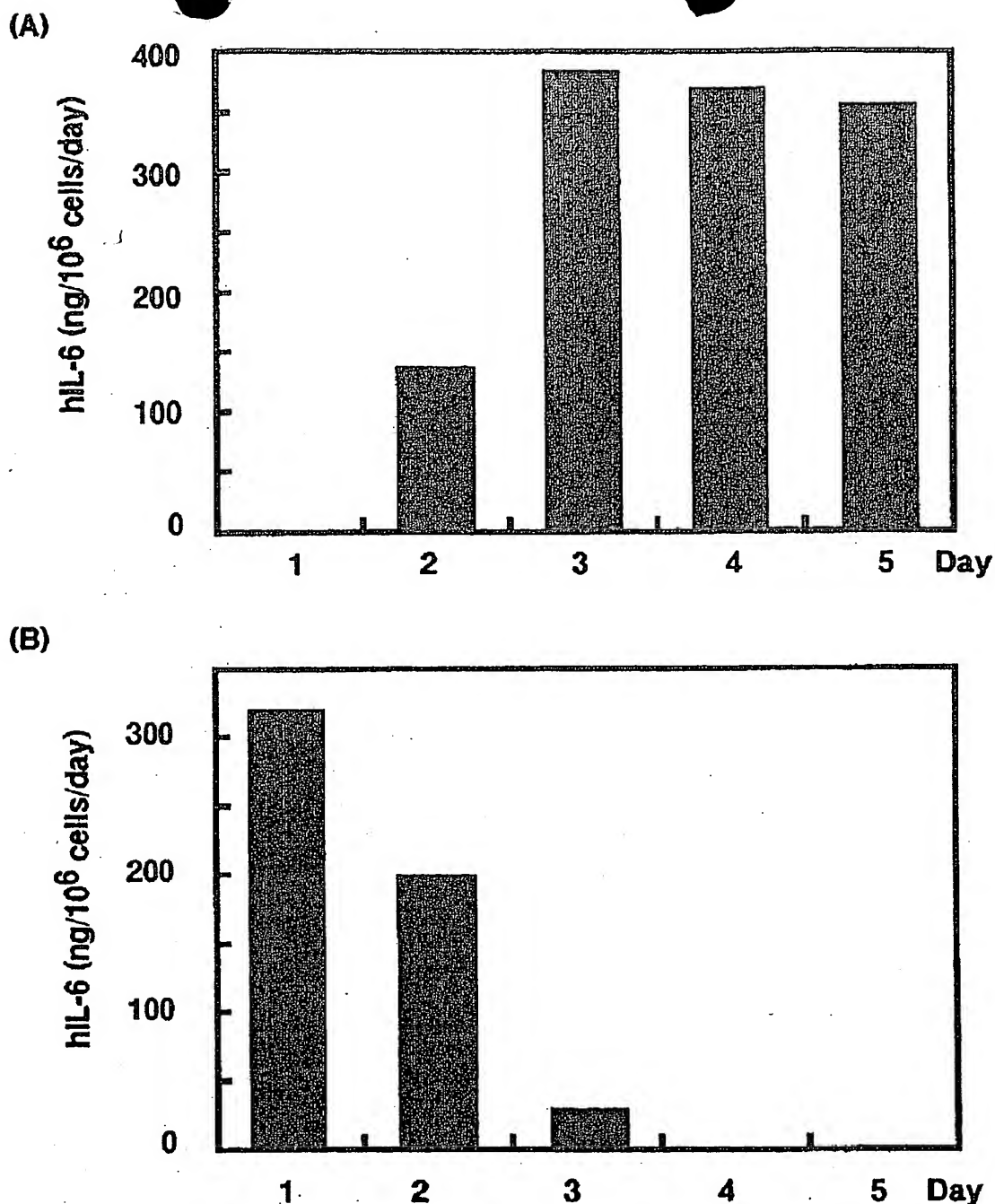


Figure 3. Inducible production of hIL-6 in UU63 clone. (A) Induction. The fully repressed cells maintained in the medium supplemented with tetracycline ($2 \mu\text{g ml}^{-1}$) were recovered by trypsin, and washed twice with PBS. The cells were then plated into five 3.5 cm diameter dishes in tetracycline free medium. Every day one dish was used to count the cell number and to determine the hIL-6 production, the remaining dishes were washed twice with PBS then maintained in fresh medium. (B) Repression. The fully induced cells were plated into tetracycline supplemented medium followed by the procedures similar to (A).

gate (Amersham, Buckinghamshire, UK). The hIL-6 was then detected using ABTS diammonium salt (Wako Chemicals, Osaka, Japan) and H_2O_2 as substrate.

Results

Efficient and inducible hIL-6 production from a VP16 transactivating domain-dependent system

As shown in Figure 1, the minimal promoter in the PhCMV*-1 can direct very low basal transcription of

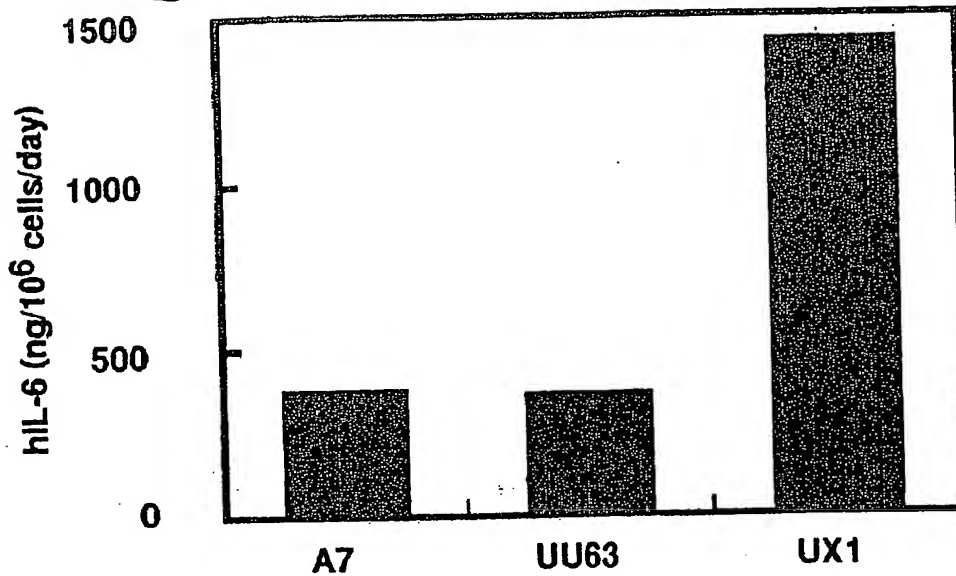


Figure 4. hIL-6 production rate in A7, UU63 and UX1 clone in tetracycline free medium. Fully induced UU63 and UX1 were used in these experiments.

the tTA gene. In the absence of tetracycline, the leaky expressed tTA proteins bind to PhCMV*-1 promoter and activate the expression of tTA gene itself through a positive feedback cycle. The transcription of the target gene is in turn strongly activated by the high cellular level of tTA transactivators.

After co-transfecting CHO cells with pUIL6-8 (Figure 2A), pUTA-1 (Figure 2B) and pSV2/bsr, we obtained sixty-one clones resistant to blasticidin S. Thirty-one of them were found to produce various levels of hIL-6 in tetracycline free medium. They were further examined to screen the clones displaying inducible production of hIL-6. One of the clones (UU63) displaying stringent regulation by tetracycline was further characterized.

Figure 3 shows the regulated production of hIL-6 in UU63 cells. The fully repressed cells was induced to produce hIL-6 after removing tetracycline. One day after induction, the hIL-6 was detected in the medium, but the level was too low to be directly quantified with ELISA. The production rate reached a peak on the third day (Figure 3A). The hIL-6 production in fully induced cells was rapidly suppressed after adding tetracycline (Figure 3B). The hIL-6 became marginally detectable after three days' suppression.

It is interesting to compare the recombinant protein production rate of this system with others using conventional strong promoter. For this purpose, we transfected the CHO cells with pCMVP-IL6 plasmid which carries a hIL-6 gene under the control of CMV promoter and a selection marker pSV2/bsr, forty-two

clones resistant to blasticidin S were obtained. Among them clone A7 displayed the highest production rate. Figure 4 shows that the fully induced UU63 clone produces hIL-6 almost as efficiently as A7 clone.

Enhancement of VP16 transactivating domain-dependent hIL-6 production by pX

We then examined whether hIL-6 production in UU63 cells could be further enhanced by the viral coactivator pX. For this purpose, we transfected UU63 cells with pX expression plasmid pECE-flagX and a selection marker plasmid pCMVD which carries a neo gene under the control of SV40 early promoter. After selecting and cloning, ten clones were expanded and their hIL-6 production rates were determined. A clone (UX1) producing significantly higher level of hIL-6 than the parent UU63 clone was further characterized. Figure 5 shows hIL-6 production in UX1 clone. As in UU63, the hIL-6 production in UX1 clone can also be induced by removing tetracycline (Figure 5A) or suppressed by adding tetracycline (Figure 5B). The hIL-6 production rate of the fully induced UX1 cells reached about 1425 ng/10⁶ cells/day, which is almost three folds higher than either of parent UU63 or A7 clone. In our experience it is not easy to achieve this production rate using conventional expression system without gene amplification.

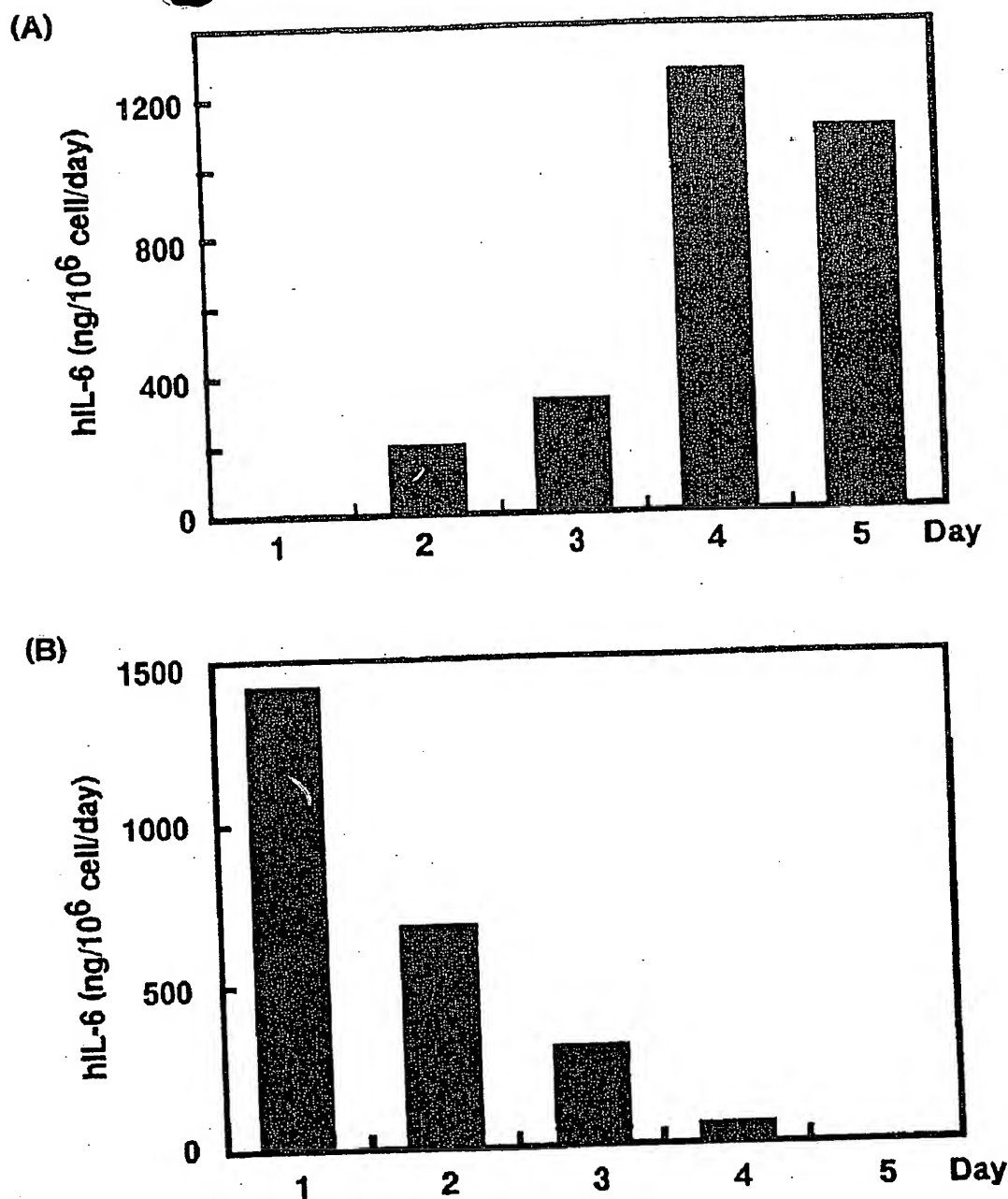


Figure 5. Inducible production of hIL-6 in UX1 clone. (A), induction. (B), repression. The experimental protocols are described in legend to Figure 3.

Discussion

A distinct feature of the system described here is that the expression of the target gene is independent of cellular regulation signals. Since the VP16 transactivating domain is capable of activating transcription from a promoter containing tetracycline operator sequences even in plant cells, the system is expected to work efficiently in a variety of eukaryotic cells.

It is very encouraging that the recombinant protein production in this system is not only stringently regulated by tetracycline but also as efficient as that conventional production system using strong promoters. Therefore, it should be a powerful tool in molecular biological study and biotechnology.

As in conventional expression system, the recombinant protein production in the tetracycline inducible system can be further enhanced by increasing the copy of the target gene (data not shown). However a poten-

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tial problem associated with gene amplification is that the basal expression will also increase proportionally. To enhance the recombinant protein production only in activated state, we tried a novel strategy, that is, using pX protein of HBV to potentiating VP16 transactivating domain. It has been reported that VP16-transactivating domain mediated transcription could be enhanced over 10 folds by pX. Like cellular coactivators, pX has little effect on the basal transcription. Furthermore, the activity of pX is not cell type specific (Haviv *et al.*, 1995). These features of pX makes it very appropriate for enhancing recombinant protein production in the tetracycline inducible expression system. In the activated state, the tTA transactivators bind to PhCMV*-1 promoter and pX enhances the transcription via VP16 transactivating domain. In the suppressed state, however, the tTA transactivators can not bind to PhCMV*-1 promoter. Without VP16 transactivating domain, pX can not activate the transcription from the PhCMV*-1 promoter. The high hIL-6 production rate and inducibility shown in UX1 cells demonstrated that this strategy is successful. It is very interesting to examine whether directly targeting the pX protein to the PhCMV*-1 promoter by fusing it to tetracycline repressor domain or to tTA transactivator can further enhance its function.

By targeting other transactivating domains or coactivators which can synergize with VP16 transactivating domain to the PhCMV*-1 promoter, we believe that it is very likely to establish a novel expression system which enables efficient and inducible production of recombinant proteins in a wide variety of eukaryotic host cells.

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Address for correspondence: Sanetaka Shirahata, Laboratory of Cellular Regulation Technology, Graduate School of Genetic Resources Technology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-81, Japan.